Maternal Care, Hippocampal Glucocorticoid Receptors, and Hypothalamic-Pituitary-Adrenal Responses to Stress

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22. Actual binding of vMIP-II to CCR3 was not determined because of the lack of success in establishing a valid binding assay with radioactively labeled eotaxin or MCP-3. However, the observation that pretreatment with vMIP-II could inhibit the calcium mobilizing effect of MCP-3 in cells expressing CCR3 indicates that vMIP-II does bind to CCR3 (Fig. 3A).
23. U87/CD4 cells expressing CCR3, CCR5, and CXCR4 were kindly provided by Dan Littman. Peripheral blood mononuclear cells (PBMCs) were prepared from buffy coat white blood cells from blood banks. After separation by density-gradient centrifugation on Ficoll-Paque, cells were cultured and stimulated with PHA. After 2 days IL-2 was added and after 3 to 5 days cells were infected. The HIV-1 strains have been described (*25, 26*). Virus stocks were prepared in PBMC cultures stimulated with PHA and IL-2. Although the same tissue culture ID_{50} (1900 for PBMC) of virus was used to challenge the cells, replication and infection were more efficient on the U87/CD4 cells expressing CXCR4 than on those expressing CCR3 or CCR5. However, lowering the amount of virus failed to substantially increase the inhibition by chemokines shown in Fig. 4 for the CXCR4-expressing cells. For determination of chemokine inhibition of infectivity, cells were seeded into 96-well dishes at 4×10^3 cells per well. On the following day chemokines were added and incubated for 30 min at 37°C before virus was added and incubated an additional 3 hours before washing three times to remove residual virus. The cells were then incubated for 5 days at 37°C before the medium was harvested and p24 concentrations were estimated (*31*). Initial time-course studies had determined that optimal production of p24 was obtained after 5 days with the virus strains that were used in the U87 cells before confluency was reached.
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32. Abbreviations for amino acids are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr.
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Maternal Care, Hippocampal Glucocorticoid Receptors, and Hypothalamic-Pituitary-Adrenal Responses to Stress

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Variations in maternal care affect the development of individual differences in neuroendocrine responses to stress in rats. As adults, the offspring of mothers that exhibited more licking and grooming of pups during the first 10 days of life showed reduced plasma adrenocorticotropic hormone and corticosterone responses to acute stress, increased hippocampal glucocorticoid receptor messenger RNA expression, enhanced glucocorticoid feedback sensitivity, and decreased levels of hypothalamic corticotropin-releasing hormone messenger RNA. Each measure was significantly correlated with the frequency of maternal licking and grooming (all r's > -0.6). These findings suggest that maternal behavior serves to "program" hypothalamic-pituitary-adrenal responses to stress in the offspring.

Several years ago Levine, Denenberg, and others (*1*) showed that the development of hypothalamic-pituitary-adrenal (HPA) responses to stress is modified by early environmental events, including infantile stimulation [or handling (*2*)]. As adults, animals exposed to brief periods of handling daily for the first weeks of life show reduced pituitary adrenocorticotropic hormone (ACTH) and adrenal corticosterone (the principal glucocorticoid in the rat) responses to stress compared with nonhandled animals (*3*). These differences are apparent as late as 24 to 26 months of age (*4*), indicating that the handling effect on HPA function persists throughout life.

Glucocorticoids act at a number of neural sites to exert an inhibitory, negative-feedback effect over the synthesis of hypothalamic releasing-factors for ACTH, notably corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) (*5*). Postnatally handled animals show enhanced glucocorticoid negative-feedback sensitivity compared with nonhandled rats (*6*) and therefore decreased hypothalamic CRH and AVP mRNA expression, as well as lower levels of both CRH and AVP immunoreactivity (*7*). The handling effect on feedback sensitivity is mediated by an increase in glucocorticoid receptor (GR) expression in the hippocampus (*8, 9*), a region that has been strongly implicated in glucocorticoid negative-feedback regulation (*10*). The increased hippocampal GR gene expression is therefore a central feature of the handling effect on HPA responsivity to stress, resulting in increased feedback inhibition of CRH and AVP synthesis and reduced pituitary ACTH release during stress.

A number of authors (*11*) have proposed that the effects of postnatal handling are mediated by changes in mother-pup interactions and that the handling manipulation itself might map onto naturally occurring individual differences in maternal care. Specifically, Levine proposed that handling of the pups altered the behavior of the mother and that these differences in mother-pup interactions then mediate the effect of handling on the development of endocrine and behavioral responses to stress. The question, then, is how this maternal mediation might occur and whether such factors might contribute to naturally occurring individual differences in HPA responses to stress.

In the Norway rat, mother-pup contact occurs primarily within the context of a nest-bout, which begins when the mother approaches the litter and gathers the pups under her; she then nurses her offspring, intermittently licking and grooming the pups (*12, 13*). Handling results in changes in mother-pup interactions (*14*). Mothers of handled pups spend the same amount of time with their litters as mothers of non-

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handled pups; however, mothers of handled litters had shorter, but more frequent, nestbouts (*15*).

We examined the behavior of mothers of handled or nonhandled litters over the first 10 days of life, a "critical" period for the handling effect on HPA development (*16*). Mothers of handled pups showed increased levels of licking and grooming of pups and arched-back nursing (LG-ABN) compared with mothers of nonhandled pups (Table 1). The frequency of these two behaviors was highly correlated ($r = +0.91$); over 90% of the instances of licking and grooming occurred while the mother was nursing her pups in the arched-back posture. Mothers of nonhandled pups nursed no less frequently than those of handled pups (*17*), but tended to more frequently adopt a "blanket" or passive posture when nursing, lying over or beside the pups. These differences in licking and grooming (and the accompanying arched-back nursing posture) were the only behaviors that served to reliably distinguish mothers of handled from those of nonhandled pups.

To determine whether the increased maternal licking and grooming affects the development of HPA responses to stress, we examined the relation between naturally occurring individual differences in maternal care and HPA development (*18*). We detected pronounced and stable individual differences in maternal licking and grooming (which again was highly correlated with arched-back nursing; $r = +0.94$). The variability among the dams in licking and grooming was substantial and of sufficient range to meaningfully study the relation between variations in postnatal maternal care and the development of adult responses to stress (*19*).

As adults, the offspring of high–LG-ABN mothers that showed significantly reduced plasma ACTH and corticosterone responses to restraint stress (*20*) compared with the offspring of low–LG-ABN mothers (Fig. 1). There were no differences in basal hormone levels (Fig. 1). These findings parallel those observed in handled versus nonhandled rats, which differ in stress-induced, but not basal HPA, activity (*3*). Moreover, the frequency of maternal licking and grooming was significantly correlated with the magnitude of the plasma ACTH ($r = -0.66$, $P < 0.01$) and corticosterone ($r = -0.65$, $P < 0.01$) responses to stress in the adult offspring. Thus, the greater the frequency of maternal licking and grooming during infancy, the lower the HPA response to stress in adulthood.

We then examined glucocorticoid feedback sensitivity in the high– and low–LG-ABN offspring by administering a bolus injection of corticosterone 3 hours before acute restraint stress (*21*). Corticosterone treatment suppressed plasma ACTH responses to restraint stress to a significantly greater extent in the high–LG-ABN offspring compared with their low–LG-ABN counterparts (75 ± 5 versus 37 ± 12%, respectively; $P < 0.01$). These findings suggest that the offspring of the high–LG-ABN mothers, like the handled animals, show increased sensitivity to the inhibitory effects of glucocorticoids on stress-induced HPA activity.

Glucocorticoid inhibition of hypothalamic CRH gene expression represents a critical feature of feedback action (*5*). Thus, we examined CRH mRNA expression (*22*) in parvocellular neurons of the paraventricular nucleus of the hypothalamus (PVNh), which send projections to the median eminence and provide the neural signal for the stimulation of ACTH release (*23*). CRH mRNA expression in the PVNh was significantly decreased in the offspring

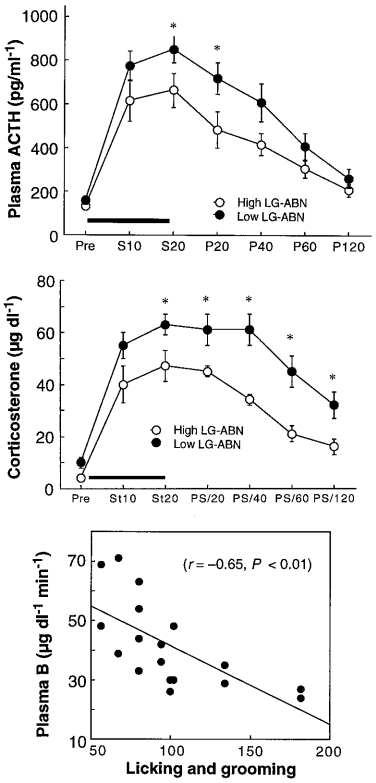

Fig. 1. Mean (±SEM) plasma ACTH (**top**) and corticosterone (middle) responses to a 20-min period of restraint stress (solid bar) in the offspring of high- versus low-licking and grooming and arched-back nursing (LG-ABN) mothers. (*) Significantly different at $P < 0.05$. Two animals from each of the nine litters were randomly choosen for testing, that is, $n = 8$ to 10 per group. (**Bottom**) Scattergram for the correlation between the frequency of maternal licking and grooming during the first 10 days of life and the integrated plasma corticosterone response to stress (calculated by use of the Trapezoidal rule).

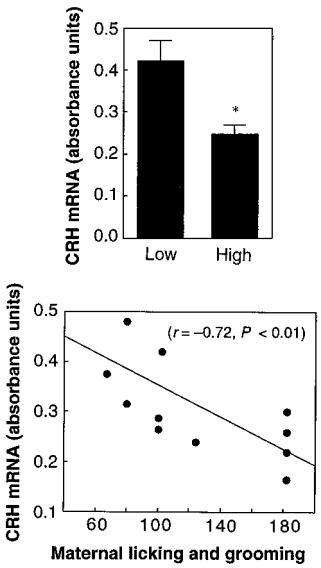

Fig. 2. (**Top**) Mean (±SEM) levels of CRH mRNA in the PVNh in the adult offspring of high– ($n = 5$) versus low–LG-ABN mothers ($n = 7$) from in situ hybridization studies of corticotropin-releasing hormone mRNA levels. CRH mRNA levels are expressed as arbitrary absorbance units. *$P <$ 0.001. (**Bottom**) Scattergram of the correlation between the frequency of maternal licking and grooming during the first 10 days of life and CRH mRNA expression in PVNh neurons in adulthood.

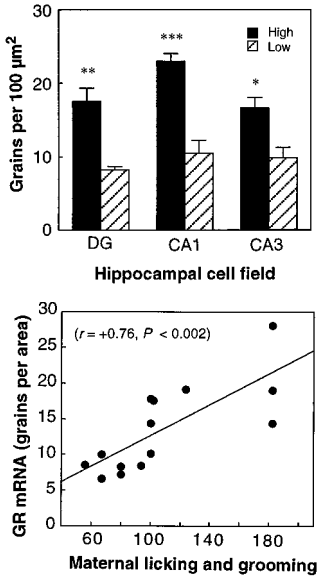

Fig. 3. (**Top**) Mean (±SEM) grains over individual cells (as a function of cell area) in dentate gyrus (DG), CA1, and CA3 cell fields of the hippocampus in adult offspring of high– ($n = 8$) versus low–LG-ABN mothers ($n = 6$)) from in situ hybridization studies of GR mRNA levels. *$P < 0.01$; **$P <$ 0.001; ***$P < 0.0001$. (**Bottom**) Scattergram of the correlation between the frequency of maternal licking and grooming during the first 10 days of life and GR mRNA expression in dentate gyrus neurons in adulthood.

of high–LG-ABN mothers compared with those of low LG-ABN mothers (Fig. 2). Moreover, CRH mRNA expression in the PVNh was significantly correlated with the frequency of maternal licking and grooming during the first 10 days of life (Fig. 2).

Considering the importance of the hippocampal GR system for negative-feedback regulation of HPA activity (*10*), we examined GR mRNA expression in the hippocampus of the offspring of high– and low–LG-ABN mothers (*24*). Across each of the hippocampal cell fields there was increased GR mRNA expression in the offspring of the high– compared to low–LG-ABN mothers (Fig. 3). Again, GR mRNA levels in each cell field of the hippocampus were significantly correlated with the frequency of maternal licking and grooming ($r = +0.76$, $P < 0.002$ for the dentate gyrus; $r = +0.64$, $P < 0.02$ for the CA1 region; $r = +0.79$, $P < 0.001$ for the CA3 region) (Fig. 3).

These findings reveal a marked similarity between the HPA responses to stress in the offspring of high–LG-ABN mothers and those of handled animals. The offspring of high–LG-ABN mothers, like handled animals, show dampened plasma ACTH and corticosterone responses to stress, increased hippocampal GR expression, enhanced glucocorticoid feedback sensitivity, and decreased hypothalamic CRH expression. There is considerable evidence for the importance of the hippocampus as a critical site for glucocorticoid feedback inhibition over hypothalamic CRH synthesis (*10*). Indeed, hippocampal GR levels have been directly correlated with CRH concentrations in the portal system of the anterior pituitary as well as with pituitary-adrenal activity (*25*). The offspring of the high–LG-ABN mothers showed increased glucocorticoid feedback sensitivity coupled with decreased hypothalamic CRH mRNA expression and, as in the handled animals, the increased hippocampal GR expression appears likely to mediate these effects.

Table 1. Mean (±SEM) number of observations (from a total of 1200) of licking and grooming in the mothers of handled or nonhandled litters (Handling study) or high– or low–LG-ABN mothers (Maternal behavior study). Differences in maternal behavior were stable over the 10-day period of observation. In neither study were there group differences in the frequency with which dams nursed pups or in pup contact (*16*). *$P < 0.01$.

Group	Licking and grooming
Handling study	
Handled	155 ± 21*
Nonhandled	78 ± 25
Maternal behavior study	
High LG-ABN	136 ± 22*
Low LG-ABN	72 ± 8

The magnitude of the HPA response to stress in adult animals was strongly correlated with maternal licking and grooming (Figs. 1 to 3). These findings support the hypothesis of Levine that the effect of postnatal handling on HPA development is mediated by effects on mother-pup interactions. Thus, handling increases the frequency of licking and grooming (Table 1) and these maternal behaviors are, in turn, associated with dampened HPA responsivity to stress (Figs. 1 to 3). Tactile stimulation derived from maternal licking and grooming regulates pup physiology and affects central nervous system (CNS) development (*26*). Variation among dams in this form of maternal behavior appears also to be associated with the development of individual differences in neuroendocrine responses to stress.

The results of the handling study suggest that the frequency of maternal licking and grooming can be regulated by stimuli associated with the pup. Thus, handling pups consistently increased maternal licking and grooming (Table 1), effectively ensuring a consistently high level of licking and grooming by the dam. This is consistent with earlier studies showing that handling increases ultrasonic vocalizations in pups which, in turn, serve to increase maternal care, including licking and grooming (*14*). However, it remains possible that the differences in maternal behavior observed here are associated with factors intrinsic to the mother—such as emotionality—in which case the data presented here may, in part, offer an example of a nongenomic mode of inheritance between parent and offspring.

We believe that the effects of early environment on the development of HPA responses to stress reflect a naturally occurring plasticity whereby factors such as maternal care are able to program rudimentary, biological responses to threatening stimuli. Like humans, the Norway rat inhabits a great variety of ecological niches, each with varied sets of environmental demands. Such plasticity could allow animals to adapt defensive systems to the unique demands of the environment. Since most mammals usually spend their adult life in an environment that is either the same as or similar to the one in which they were born, developmental "programming" of CNS responses to stress in early life is likely to be of adaptive value to the adult (*12*, *27*). Such programming affords the animal an appropriate HPA response, minimizing the need for a long and perhaps unaffordable period of adaptation in adult life. Our results suggest that this neonatal programming occurs via the differentiation of the GR system in forebrain neurons that govern HPA activity in response to variations in maternal behavior.

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2. The handling procedure involves removing the mother and then rat pups from their cage, placing the pups together in a small container, and returning the animals 15 min later to their cage and their mothers. The manipulation is generally performed daily for the first 21 days of life. Handling does not represent a period of maternal deprivation, because over the course of the day mothers are routinely off their nests and away from pups for periods of 20 to 25 min. At the same time, the artificial and nonspecific nature of the handling paradigm is unsettling [M. Daly, *Br. J. Psychol.* **64**, 435 (1972)]. Normal development in a rat pup most often occurs in the rather dark, tranquil confines of a burrow where the major source of stimulation is the mother and littermates.
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Nonhandled animals were completely undisturbed until day 12 of life at which time normal cage maintenance was initiated. The behavior of each dam was observed [M. M. Myers, S. A. Brunelli, H. N. Shair, J. M. Squire, M. A. Hofer, *Dev. Psychobiol*. **22,** 55 (1989)] for eight 60-min observation periods daily for the first 10 days after birth with six periods during the light phase and two periods during the dark phase of the L:D cycle. The distribution of the observations was based on the finding that nursing in rats occurs more frequently during the light phase of the cycle. Handling occurred each day at 1100, and an observation was scheduled at 1130 to correspond to the reunion of the mothers and pups. Within each observation period the behavior of each mother was scored every 4 min (15 observations per period × 8 periods per day = 120 observations per mother per day) for mother off pups, mother licking and grooming any pup, or mother nursing pups in either an arched-back posture, a "blanket" posture in which the mother lies over the pups, or a passive posture in which the mother lies either on her back or side while the pups nurse. Behavioral categories are not mutually exclusive.

17. D. Liu *et al.*, data not shown.
18. Nine Long-Evans female rats were mated in our animal facility and housed and observed as described (*16*). The animals underwent routine cage maintenance beginning on day 12 but were otherwise not manipulated. At the time of weaning on day 22 of life, the male offspring were housed in same-sex, same-litter groups. Testing of offspring occurred no earlier than 100 days of age.
19. We then rank-ordered the dams on licking and grooming, identifying those mothers whose scores fell above the mean, and as a group these dams were classified as high LG-ABN. The remaining dams were classified as low LG-ABN. The offspring were tested beginning at 100 days of age.
20. For restraint stress (20 min) testing (*6*), two animals from each of the nine litters were randomly selected for testing. Blood samples were collected from indwelling right jugular vein catheters (*6*), implanted 4 days before restraint stress testing, and replaced with an equal volume of normal saline (0.9%) via the same route. We have found that by 72 hours after surgery, basal ACTH and corticosterone levels have returned to normal (*6*). Plasma corticosterone was measured by radioimmunoassay [L. C. Krey *et al.*, *Endocrinology* **96,** 1088 (1975)]. Plasma (25 μl) ACTH was measured by radioimmunoassay as described [C.-D. Walker, S. F. Akana, C. S. Cascio, M. F. Dallman, *ibid.* **127**, 832 (1990); V. Viau and M. J. Meaney, *ibid.* **129**, 2503 (1991)]. All samples were run within a single assay. In our lab the intra- and interassay coefficients of variation are 7 and 10%, respectively, for corticosterone and 8 and 11% for ACTH. The data were analyzed by two-way analyses of variance (ANOVA) with one between (group) and one within (sample) measure. Post hoc analysis was performed by Tukey test.
21. We used a delayed negative-feedback paradigm [M. Keller-Wood and M. F. Dallman, *Endocr. Rev.* **5**, 1 (1984)] in which animals are steroid-treated 2 to 4 hours before acute stress. The animals used in this study were the same animals prepared with jugular catheters for acute restraint testing. The animals were tested 4 days after restraint stress, and all but one of the catheters remained patent during this interval. The critical measure here is the ability of the steroid to inhibit subsequent HPA responses to stress. Animals were injected subcutaneously with either vehicle alone or a low to moderate dose of corticosterone (1 mg/kg in ethanol:saline/1:9) on the basis of earlier studies (*6*) showing that this dose discriminates feedback sensitivity in handled versus nonhandled rats. Restraint stress was done as described above and plasma samples were obtained from jugular catheters immediately before and at the end of the 20-min period of restraint, a time point that corresponds to the peak plasma ACTH level (*6*). The percentage suppression of plasma ACTH responses to stress for the high– versus low–LG-ABN groups was derived by comparing Δ(peak stress level − basal level) for each of the corticosterone-treated animals in both groups with that of the mean for the respective control groups (vehicle-treated high– or low–LG-ABN animals). Percentage suppression scores were used to accommodate for the groups differences in plasma ACTH responses to acute stress. The results were examined statistically by Mann-Whitney U test on the basis of percentage scores.
22. CRH mRNA in situ hybridization was done with a 48–base pair (bp) oligonucleotide sequence (CAGTTTCCTGTTGCTGTGAGCTTGCTGAGCTA-ACTGCTCTGCCCTGGC) (Perkin-Elmer,Warrington, UK) and a modified version of the procedure previously described [N. Shanks, S. Larocque, M. J. Meaney, *J. Neurosci.* **15,** 376 (1995)] with brain sections obtained from animals rapidly killed under resting-state conditions. After hybridization, sections were apposed to Hyperfilm (Amersham) for 21 days along with sections of ^{35}S-labeled standards prepared with known amounts of radiolabeled ^{35}S in a brain paste. The hybridization signal within the parvocellular subregion of the PVNh was quantified by densitometry with an MCID image analysis system (Imaging Research, St. Catherine's, Ontario, Canada). The data are presented as arbitrary absorbance units after correction for background. These data were analyzed by *t* test for unpaired groups.
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24. GR in situ hybridization was done as described [(*9*); J. R. Seckl, K. L. Dickson, G. Fink, *J. Neuroendocrinol.* **2**, 911 (1990)] with [^{35}S]UTP-labeled cRNA antisense probes transcribed with T7 RNA polymerase from a 674-bp Pst I–Eco RI fragment of the rat GR cDNA linearized with Ava I. After hybridization, sections were dehydrated, dried, and dipped in photographic emulsion (NTB-2, Kodak), and then stored at 4°C for 21 days before development and counterstaining with Cresyl Violet. The hybridization signal within dorsal hippocampal subregions was quantified by grain counting within high-power microscopic fields under brightfield illumination. Grain counting was performed by an individual unaware of the group from which the slide was derived. For each cell field, grains over ~40 to 50 individual neurons per section were counted, on three sections per animal (*9*). After subtraction of background (grains over neuropil), mean values were derived for each hippocampal cell field for each animal. Background ranged between 10 and 15% of values found over hippocampal cells. Grain counts are presented as a function of cell area to account for possible morphological differences [J. T. McCabe, R. A. Desharnais, D. W. Pfaff, *Methods Enzymol.* **168**, 822 (1989)]. These data were analyzed by two-way ANOVA with one between measures (group) and one repeated measure (hippocampal sub-field) by Tukey post hoc test.
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Structure of a Murine Leukemia Virus Receptor-Binding Glycoprotein at 2.0 Angstrom Resolution

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An essential step in retrovirus infection is the binding of the virus to its receptor on a target cell. The structure of the receptor-binding domain of the envelope glycoprotein from Friend murine leukemia virus was determined to 2.0 angstrom resolution by x-ray crystallography. The core of the domain is an antiparallel β sandwich, with two interstrand loops forming a helical subdomain atop the sandwich. The residues in the helical region, but not in the β sandwich, are highly variable among mammalian C-type retroviruses with distinct tropisms, indicating that the helical subdomain determines the receptor specificity of the virus.

Retroviruses are simultaneously a profound human medical problem and a potential medical solution. They can be pathogenic, causing immunodeficiency, leukemia, and neurological disease, but they are also actively studied for their proposed utility as gene therapy vectors. Essential to both roles is the targeting of the virus to the host cell through interactions between viral envelope proteins and cell surface proteins.

Retrovirus envelope glycoproteins (*1*) are synthesized as single chain precursors that are subsequently cleaved into two subunits, the surface (SU) and the transmembrane (TM) (Fig. 1). The SU glycoprotein binds the receptor. The TM subunit contains the hydrophobic fusion peptide and transmembrane segments and is likely to participate directly in the fusion of the viral and cellular membranes after receptor binding.

Efforts to understand the structural basis of retroviral binding and entry and to devel-